Volume 247, Number 1, February 25, 1999

50 Years ECR 1949-1999

Experimental Cell Research

Academic Press

Experimental Cell Research 247, 200–207 (1999)
Article ID excr.1998.4349, available online at http://www.idealibrary.com on IDEAL®

Overexpression of Anti-apoptotic Gene BAG-1 in Human Cervical Cancer

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Apoptosis is a programmed cell death process in which cells commit suicide under certain environmental conditions. Recent studies suggest that apoptosis is controlled by a variety of cellular genes, and dysregulation of these genes plays an important role in the pathogenesis of human diseases, including cancer. BAG-1 is a novel anti-apoptotic protein isolated by its interaction with another anti-apoptotic protein, Bcl-2. It binds to several hormone receptors and growth factor receptors and modulates their function in apoptosis. However, the role of BAG-1 in the oncogenesis of human cervical cancer has yet to be illustrated. In this study, we examined the expression of BAG-1 in cervical normal and carcinoma cultured cells and tissues. BAG-1 was overexpressed in human cervical carcinoma cell lines and tissues. Overexpression was regulated at the transcriptional level. The increased expression of BAG-1 was correlated with enhanced resistance of cervical carcinoma cells to apoptosis induced by a DNA-damaging reagent. In addition, overxpression of BAG-1 enhanced the resistance of cervical cells to apoptosis. This study provided the first evidence that BAG-1 is upregulated in human cervical cancer and may play an important role in apoptosis and human cervical carcinogenesis. © 1999 Academic Press

INTRODUCTION

Cervical cancer is the second most frequent malignancy among women in the world [1]. In the past 10 years, compelling evidence confirmed the hypothesis that human papillomaviruses (HPVs), especially high risk HPVs such as HPV16 and HPV18, are the major cause of this cancer. Furthermore, *in vitro* and *in vivo* studies strongly suggest that HPV infection is necessary but not sufficient for causing cervical cancer. Other factors, such as hormones, cigarette smoke, and dysregulation of oncogenes and tumor suppressor genes, are also required for full malignant transformation [2–4]. However, the molecular mechanism of the multistep, multifactor oncogenesis of cervical cells remains unclear.

Apoptosis or programmed cell death is a ubiquitous process in which cells commit suicide under certain environmental conditions. A variety of studies suggest that apoptosis is controlled through several cellular genes including inducers (*p53, bad, bax, bak, blc*-X_S, and *bik*) and inhibitors (BAG-1, *bcl*-2, *bcl*-X_L, and *mcl*-2) of apoptosis [5–10]. It has been shown that p53, an inducer of apoptosis, is deleted or mutated in many cases in a variety of human malignancies. In contrast, Bcl-2, an inhibitor of apoptosis, is overexpressed in many different human tumors [11–17]. These findings indicate that alterations in apoptosis-regulating genes play important pathological roles in cancer [for review, see 18–20]. Recently, overexpression of Bcl-2 was found in cervical carcinoma cell lines and premalignant and malignant tissues [16, 21, 22], suggesting that enhanced level of anti-apoptosis proteins may underlie the development of cervical cancer. However, the expression of apoptosis-associated proteins other than Bcl-2 and p53 has not been examined in cervical cancer.

BAG-1 is an anti-apoptotic protein that was isolated by virtue of its interaction with Bcl-2 [9]. It not only inhibited apoptosis independently but also enhanced the anti-apoptotic activity of Bcl-2 [9]. In addition, BAG-1 forms complexes with a number of hormones and growth factor receptors, such as those for glucocorticoid, estrogen, hepatocyte growth factor, and platelet-derived growth factor, and modulates their ability in apoptosis [23–26]. Therefore, BAG-1 was regarded as a multifunctional anti-apoptotic protein capable of interacting with various cellular proteins.

Despite extensive studies of the interactions of BAG-1 with other proteins, the potential function of BAG-1 in the carcinogenesis of human cancers remains to be elucidated. In this report, we investigated the expression of BAG-1 in cervical oncogenesis. The re-

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0014-4827/99 $30.00

sults show that both BAG-1 RNA and protein were overexpressed in human cervical carcinoma cell lines and tissues, indicating that BAG-1 may play an important role in the development of cervical cancer.

MATERIALS AND METHODS

Containment for biohazards. Level 2 biohazard containment procedures recommended by the MRC of Canada were followed for all tissue culture and experiments using human cervical biopsies. The experiments were approved by the Memorial University of Newfoundland Biosafety Committee.

Cell culture and establishment of BAG-1-overexpressing transfectant. Human endocervical cells (HEN) and ectocervical cells (HEC) were obtained after dissection of pathologically normal cervical tissues derived from hysterectomies performed for benign conditions and were maintained in serum-free medium for keratinocytes (GIBCO BRL, Bethesda, MD). All cervical carcinoma cell lines were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

For stable transfection of BAG-1 into cervical carcinoma C33A cells, a plasmid called p50K was constructed by cloning full-length BAG-1 cDNA (p50) containing a Kozak sequence into the PCR3.1 eukaryotic expression vector. The transfection of p50K (BAG-1) and a control PCR3.1 plasmid (NEO) into C33A and the establishment of a transfectant line was as described [27].

Apoptosis assays. To assay apoptosis in cervical cells induced by a DNA-damaging reagent, staurosporine, exponentially growing cells were seeded at 5×10^4 cell/well in 12-well plates and incubated for 24 h. Then the cells were treated with either 0.2 μM staurosporine for the indicated times for the experiments comparing primary and cancer cells or 0.5 μM staurosporine for the indicated times for the experiments with stably BAG-1-transfected C33A cells. Cell viability was determined with the trypan blue exclusion assay.

RNA and protein extraction from human cervical cell lines or biopsies. Total RNA was extracted from cervical normal and carcinoma cells at 70% confluence using the CsCl gradient centrifugation method [28]. Protein was extracted from 10^7 cells or 0.1-g biopsies by lysis in 1 ml ice-cold extraction buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 100 μg/ml PMSF, 50 μg/ml aprotinin) for 30 min and centrifugation at 12,000g for 10 min.

Northern, Western, and Southern blot analysis. Northern blot and Western blot analysis of cellular gene expression was as described [4] with minor modifications. In brief, for Northern blot, 20 μg of total RNA was separated in denaturing 1% agarose gels and transferred to Hybond nitrocellulose membranes (Amersham, Bethesda, MD). The blots were hybridized with ^{32}P-labeled human BAG-1 cDNA in Rapid-Hyb hybridization buffer (Amersham) at 65°C for 1 h, washed twice with 2× SSC/0.1% SDS at room temperature for 15 min and twice with 0.1× SSC/0.1% SDS at 65°C for 10 min, and then exposed to Kodak BioMax film overnight. For Western blot analysis, 10 μg of protein was fractionated by 10% SDS–PAGE and transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham) under semidry conditions. Immunodetection was done using the ECL system (Amersham), according to the manufacturer's instructions. Anti-BAG-1 monoclonal antibody (mAb) was generated as described [27]. Anti-Bcl-2 and anti-β-actin mAbs, and anti-Bcl-X_L and anti-GR polyclonal antibodies, were purchased from Santa Cruz, Inc. (Santa Cruz, CA).

DNA extraction and Southern blot analysis of high-molecular-weight genomic DNA from cervical cells was as described [29].

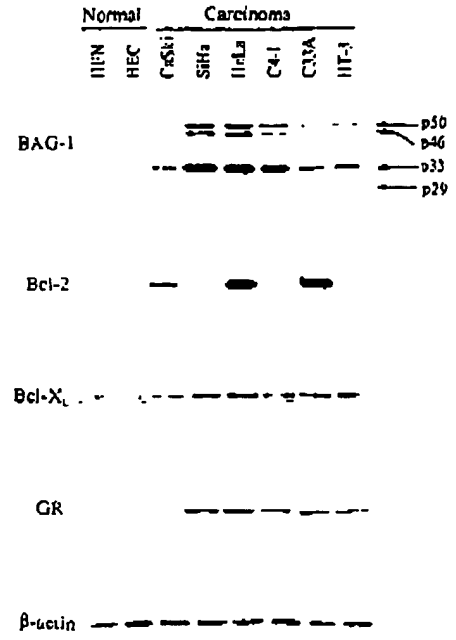

FIG. 1. Overexpression of BAG-1 and its interacting proteins in cervical carcinoma cell lines. For Western blots, 10 μg of proteins from normal HEN and HEC and cervical carcinoma cells was separated in a 10% SDS–PAGE gel and transferred to an ECL nitrocellulose membrane. Each protein was detected by the ECL system using primary antibodies to BAG-1, Bcl-2, Bcl-X_L, and GR and then secondary HRP-conjugated anti-mouse IgG. β-Actin was used as an internal control.

RESULTS

Overexpression of BAG-1 Protein in Cervical Carcinoma Cell Lines and Tissues

We first examined the expression of BAG-1 protein in normal and cervical carcinoma cell lines using Western blot analysis to determine if BAG-1 is involved in the oncogenesis of cervical cells. While no BAG-1 protein was detected in primary HEN and HEC, three (p50, p46, and p33) of the four isoforms of BAG-1 w re overexpressed in all the cervical carcinoma cell lines. The shortest isoform of BAG-1, p29, was expressed only in SiHa and HeLa cells, in which the highest protein level of p50, p46, and p33 was found (Fig. 1). Since synergistic effect of BAG-1 and Bcl-2 in anti-apoptosis was found [9], we examined whether the levels of BAG-1-interacting proteins Bcl-2, Bcl-X_L, and GR were also enhanced in cervical carcinoma cell lines. As was seen for BAG-1, no or low expression of Bcl-2, Bcl-X_L, and GR was found in primary HEN and HEC, whereas Bcl-2, Bcl-X_L, and GR were overexpressed in three (CaSki, HeLa, and C33A), five (SiHa, HeLa, C4-I, C33A, and HT-3), and six (CaSki, SiHa, HeLa, C4-I,

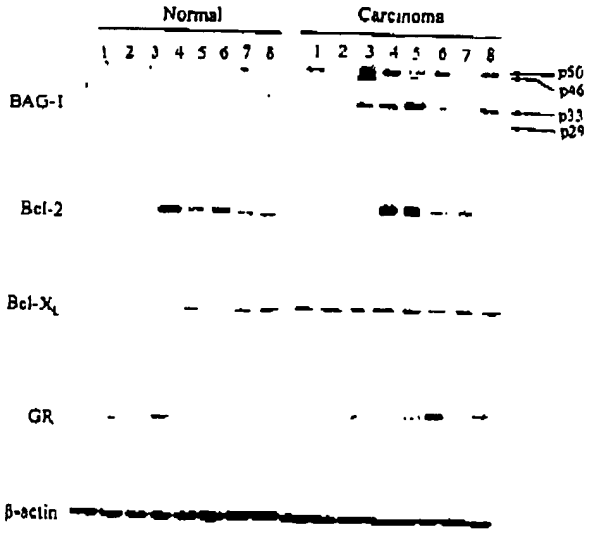

FIG. 2. Overexpression of BAG-1 protein in cervical carcinoma tissues. Western blot methods and labels were as described for Fig. 1. Each lane, numbered 1–8, represents a different cervical normal or carcinoma tissue.

C33A, and HT-3), respectively, of the six cervical carcinoma cell lines (Fig. 1). The expression levels of Bcl-X_L and GR were correlated with that of BAG-1. However, the expression levels of Bcl-2 and BAG-1 were not correlated. For example, the highest expression of BAG-1 was found in SiHa cells, whereas no Bcl-2 was detected in this cell line. On the other hand, the lowest level of BAG-1 protein was found in C33A cells, while the highest level of Bcl-2 was detected in this cell line (Fig. 1).

To compare our results for cell lines with those *in vivo*, we examined the expression of BAG-1 and its interacting proteins in primary normal and cervical carcinoma tissues. In general, BAG-1 protein was low or not detected in normal cervical tissues, but it was overexpressed in most of the cervical carcinomas. Each isoform of BAG-1 displayed a distinct pattern in these tissues. p50 was detected in only two of eight (25%) normal cervical tissues, whereas it was overexpressed in six of eight (75%) cervical carcinomas; p46 was not expressed in any of the normal tissues, whereas it was detected in two of eight (25%) cervical carcinomas; p33 was not present in any of the normal tissues, whereas it was overexpressed in seven of eight (88%) cervical carcinomas; p29 was not detected in any of the tissues examined (Fig. 2). On the other hand, in contrast to the cultured cells, all three BAG-1-interacting proteins, Bcl-2, Bcl-X_L, and GR, were expressed on average at comparable levels in both cervical normal and carcinoma tissues (Fig. 2).

RNA Expression and Integrity of the BAG-1 Gene in Cervical Normal and Carcinoma Cell Lines

To investigate whether the overexpression of BAG-1 protein in various cancer cell lines is caused by enhanced transcription, we analyzed the expression of BAG-1 RNA from cervical cultured normal and cancer cells by Northern blot. Our experiment showed that the expression of BAG-1 RNA correlated well with that of BAG-1 protein (Figs. 1 and 3), suggesting that th overexpression of BAG-1 protein is caused by increased transcription of BAG-1 gene.

Since high level of *bcl*-2 expression in follicular lymphoma is thought to be a consequence of the t(14;18) chromosomal translocation of the *bcl*-2 gene [30], we used Southern blot analysis to examine whether a genomic rearrangement might underlie the increased transcription of BAG-1 RNA in cervical carcinoma cells. When DNA from cervical normal and carcinoma cells was digested with *Eco*RI, four BAG-1 DNA fragments with molecular weights of 2.0, 3.0, 8.1, and 15.2 kb were present from all the cell types. When the DNA was cleaved with *Pst*I, four BAG-1 DNA fragments with molecular weights of 0.7, 1.8, 5.0, and 10.0 kb were found from all the samples. Compared to the band pattern of HEN and HEC DNA, no loss or gain of BAG-1 bands was detected in any of the cervical carcinoma cell lines (Fig. 4).

Effect of BAG-1 Overexpression on the Resistance of Cervical Carcinoma Cells to Apoptosis Induced by Staurosporine

BAG-1 was originally identified as an anti-apoptotic protein [9]. Therefore, we examined whether enhanced resistance to apoptosis in cervical carcinoma cells was correlated with the overexpression of BAG-1. As ex-

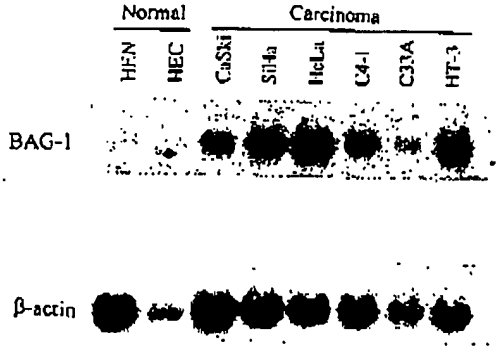

FIG. 3. Overexpression of BAG-1 RNA in cervical carcinoma cell lines. For Northern blots, 20 μg of total RNA from normal HEN and HEC and cervical carcinoma cells was size-fractionated on a 1.0% agarose formaldehyde gel, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled BAG-1 cDNA probe. β-Actin was used as an internal control.

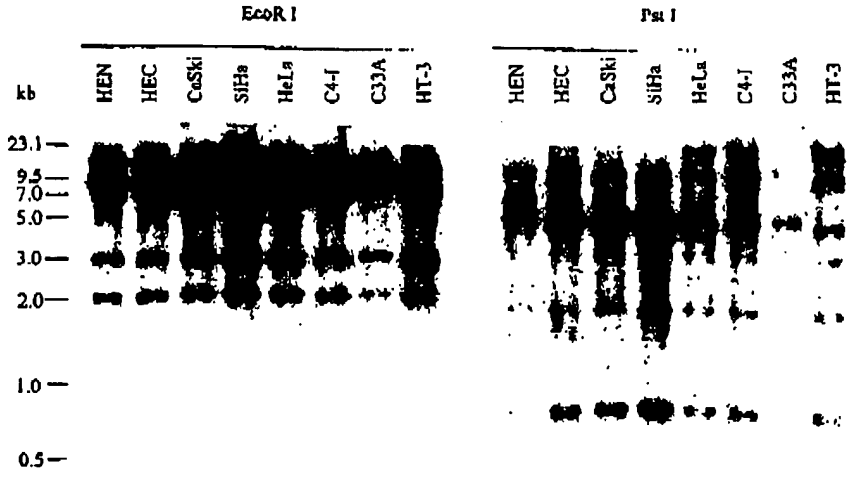

FIG. 4. Southern blot analysis of BAG-1 in genomic DNA from cervical normal and carcinoma cells. Ten micrograms of high-molecular-weight genomic DNA was digested with *Eco*RI or *Pst*I, separated in a 1% agarose gel, and transferred to a Hybond-NX nylon membrane. BAG-1 DNA was detected by probing the blot with ^{32}P-labeled full-length human BAG-1 cDNA. Molecular weight markers are shown on the left.

pected, decreased cell viability or enhanced resistance to apoptosis correlated well with overexpression of BAG-1 p50. As shown in Fig. 5, while all the cells underwent apoptosis after treatment of HEN and HEC with 0.2 μM staurosporine for 24 h, the viability of cervical carcinoma cells (CaSki, SiHa, HeLa, C4-I, C33A, and HT-3) was over 80% after 48 h treatment.

To examine whether BAG-1 is involved directly in the resistance of cervical carcinoma cells to apoptosis, we stably transfected BAG-1 cDNA into the C33A cervical carcinoma cell line, which expressed low level of BAG-1 (Fig. 1). Since C33A was relatively resistance to apoptosis induced by 0.2 μM staurosporine, we assayed apoptosis after treatment of the cells with 0.5 μM staurosporine. Because distinct isoforms of human BAG-1 have similar functions in anti-apoptosis (unpublished data), only the result for p50 is shown in Fig. 6. The viability was significantly higher in the cells overexpressing BAG-1 (BAG-1) than in the cells expressing vector plasmid (NEO) after treatment with 0.5 μM staurosporine for 6 days ($P < 0.05$; Fig. 6), indicating that overexpression of BAG-1 enhanced the resistance of cervical cells to apoptosis.

DISCUSSION

A number of studies support the concept that HPVs, a family of small double-stranded circular DNA viruses, are the major cause of cervical cancer [31–33]. High-risk HPVs, in particular HPV16 and HPV18, have been detected in 90% of cervical carcinoma biopsies [33, 34]. Since cooperation of the E6 and E7 proteins of high-risk HPVs is sufficient to immortalize human cervical keratinocytes and epithelial cells, they have been considered oncogenic proteins important for cervical carcinogenesis [for review, see 32, 35]. E6 and E7 proteins were shown to bind to tumor suppressor proteins p53 and Rb, respectively, and to inactivate their tumor suppressor activity, leading to uncontrolled cell growth [35–38]. Therefore, inactivation of p53 and Rb by HPV16 E6 and E7 was considered one of the necessary steps toward transformation of cervical cells.

p53 is a tumor suppressor gene that functions as a participant in cell cycle control, DNA synthesis and repair, and maintenance of genomic stability [for review, see 39]. Deletions or mutations of *p53* have been detected in about 50% of human cancers [12, 39]. Recently, *p53* was also found to play an important role in apoptosis. Loss of p53 function resulted in resistance to apoptosis induced by DNA-damaging reagents in various human cells [40]. On the other hand, overexpression of *p53* was shown to induce apoptosis in certain cell types [41–43]. Although the exact role of p53 in apoptosis has not been fully elucidated, accumulating data suggest that regulation by p53 of cellular genes associated with apoptosis is important for its proapoptotic function. In support of this suggestion, it was recently reported that p53 can modulate apoptosis by upregulating the expression of the proapoptotic gene *bax* and downregulating the anti-apoptotic gene *bcl-2* [44–46]. Since p53 was inactivated in a variety of human cancers, it was consistent that enhanced expression of Bcl-2 was observed in these cancers [11–17, 47]. Also, reduced expression of *bax* RNA was found in breast cancer cell lines and tissues [48, 49]. Overall,

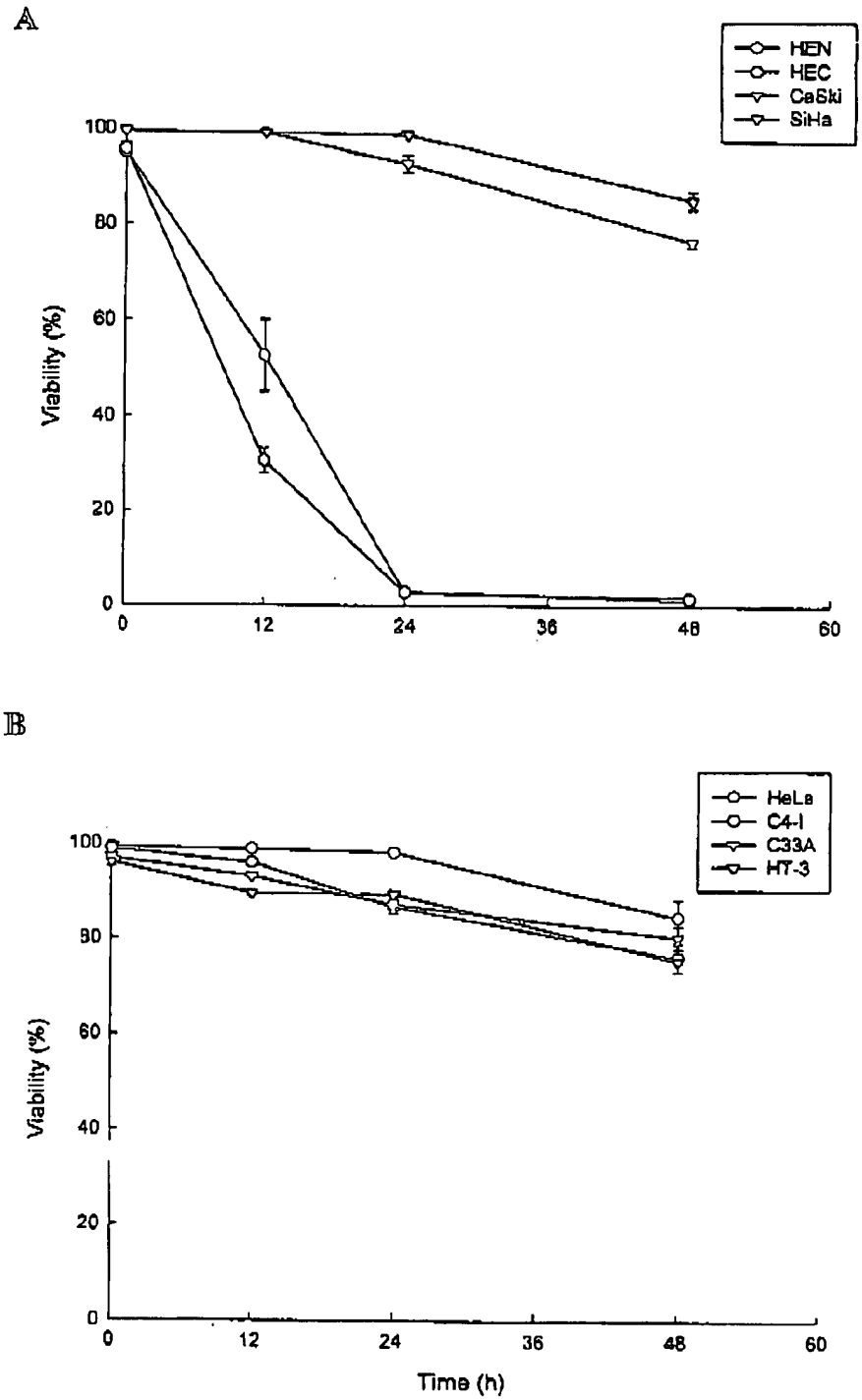

FIG. 5. Resistance of human cervical carcinoma cells to apoptosis induced by staurosporine. Cervical normal (A) and carcinoma (A and B) cells were treated with 0.2 μM staurosporine for the indicated times. The percentage of viable cells was determined by trypan blue exclusion assays. The results represent the means ± the standard deviation of three experiments.

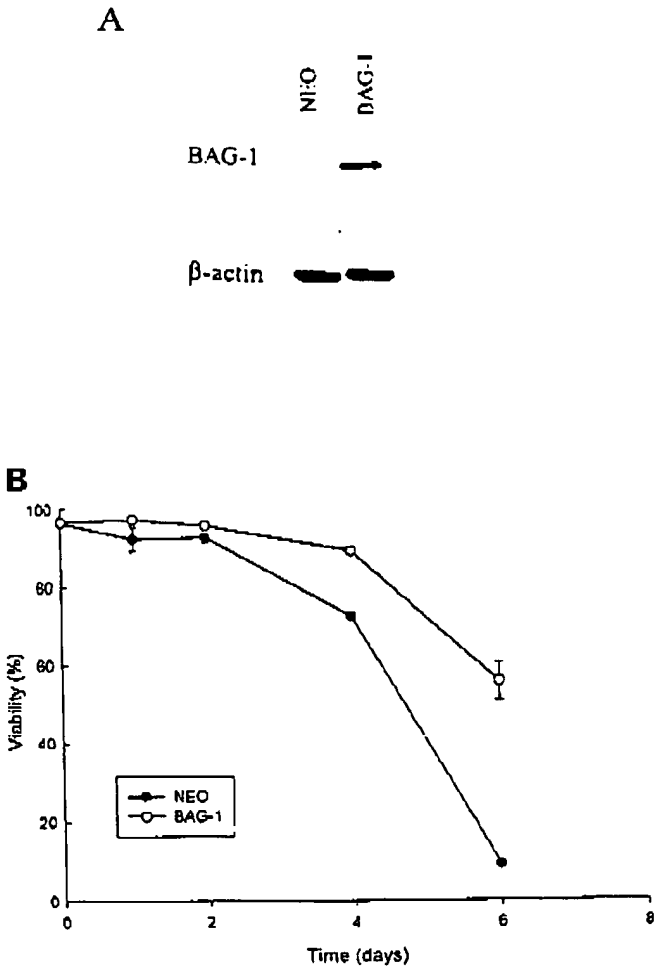

FIG. 6. Enhanced resistance to staurosporine-induced apoptosis by overexpression of BAG-1 cDNA in C33A cells. The vector control plasmid PCR3.1 (NEO) and the plasmid containing p50K cDNA (BAG-1) were stably transfected into C33A cells. (A) Overexpression of BAG-1 p50 in C33A cells. Western blot methods and labels were as in Fig. 1. (B) Resistance to apoptosis induced by staurosporine in BAG-1-overexpressing C33A cells. The cells were treated with 0.5 μM staurosporine for the indicated times. Detection of apoptosis and presentation of the data are as described for Fig. 5.

these results suggested that upregulation of anti-apoptotic genes and downregulation of proapoptotic genes may be important in oncogenesis.

Except for *p53* and *bcl-2*, expression of apoptosis-associated genes has not been extensively examined in cervical cell lines and tissues. In this study, we provided the first evidence that anti-apoptotic protein BAG-1, a Bcl-2-interacting protein, is overexpressed in human cervical carcinoma cell lines and tissues and that overexpression is due to increased transcription of the BAG-1 gene. On the other hand, overexpression of BAG-1-interacting proteins Bcl-2, Bcl-X_L, and GR was found in only cervical carcinoma cell lines and not cervical carcinoma tissu s. Enhanced expression of Bcl-2 in cervical cancer cell lin s and tissues has also been reported previously [21, 22, 50]. One likely explanation for the discrepancy between our results and those of others for the expression of Bcl-2 in cervical carcinoma tissues is that all the previous studies used immunocytochemistry in which the level of Bcl-2 represented the percentage of cells stained but not the total signal intensity from all the cells as presented in our data using Western blot analysis. However, our results suggest that BAG-1 may serve as a better molecular marker than Bcl-2, Bcl-X_L, or GR for the oncogenesis of cervical cells *in vivo*. Because only eight normal and eight cancerous samples were used in this study, analysis of a larger number of samples will be performed to address this suggestion.

The exact mechanism for the enhanced expression of BAG-1 in cervical cancers is unknown. Since p53 was inactivated by either its mutation (C33A and HT-3) or HPV (CaSki, SiHa, HeLa, and C4-I) in cervical carcinoma cell lines [51], the enhanced expression of BAG-1 may be the result of loss of transcriptional repression by p53. Consistent with this hypothesis, enhanced x-pression of BAG-1 was also found in HPV16-immortalized HEN in which p53 was reduced [52], probably through its degradation by HPV16 E6. We have recently cloned the promoter region of the BAG-1 gene. A sequence motif for negative regulation by p53 has b en found in the BAG-1 promoter (unpublished data). We are currently testing whether this sequence is functional for the repression of BAG-1 transcription by p53.

Although the role of BAG-1 in apoptosis has been extensively studied, it had not been examined in cervical cells. This study provided the first evidence that overexpression of BAG-1 in cervical carcinoma cells may partially contribute to their enhanced resistance to apoptosis induced by DNA-damaging reagents. The enhanced resistance to induction of apoptosis in cervical carcinoma cells is not due to a reduced expression of the proapoptotic proteins Bax and Bak, since no difference in Bax and Bak expression was found compared with that in HEN and HEC (unpublished data). Although the increase of Bcl-X_L in cervical carcinoma cells was not pronounced compared with that in normal cervical cells, enhanced expression of Bcl-X_L may also contribute to the enhanced resistance of cervical carcinoma cells to apoptosis. In addition, our results suggest that BAG-1 and Bcl-2 have important independent compensatory effects in apoptosis, apart from the synergistic effect found by Takayama *et al.* [9]. For example, overexpression of BAG-1 but not Bcl-2 was found in SiHa cervical carcinoma cells that had enhanced resistance to apoptosis. On the other hand, much higher level of Bcl-2 than BAG-1 was detected in

C33A cervical carcinoma cells that also exhibited enhanced resistance to apoptosis.

In conclusion, our study provided the first evidence that BAG-1 protein is overexpressed in human cervical carcinoma cell lines and tissues. Reduced apoptosis in cancer cells by upregulation of BAG-1 possibly plays an important role in the development of cervical cancer.

We thank G. Chernenko for excellent technical assistance. The investigation was supported by grants from the Medical Research Council of Canada, a grant from the National Cancer Institute of Canada with funds from the Canadian Cancer Society, and a grant from the Newfoundland Cancer Treatment and Research Foundation.

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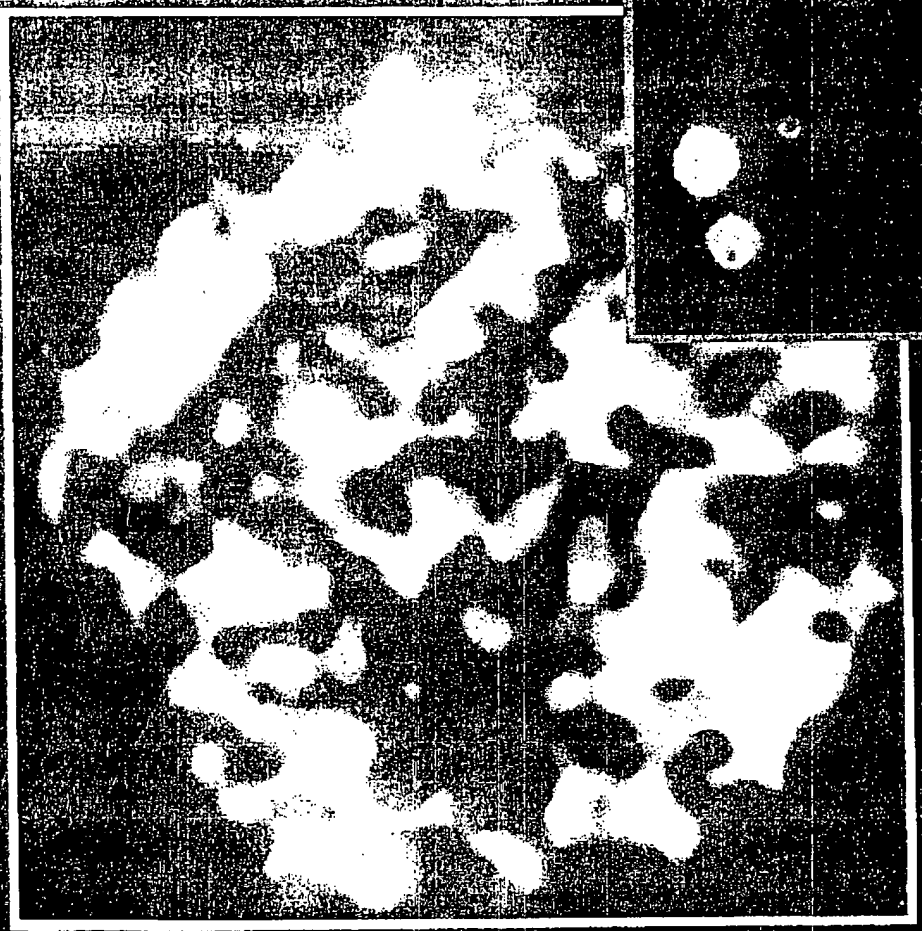
Received November 17, 1998

ISSN 0014-4827
Volume 263, Number 2, May 1, 2000
This Number Complete Volume 263

Experimental Cell Research



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Experimental Cell Research 256, 583 (2000)
doi:10.1006/excr.2000.4890, available online at http://www.idealibrary.com on IDEAL®

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Volume 247, Number 1 (1999), in the article "Overexpression of Anti-apoptotic Gene BAG-1 in Human Cervical Cancer," by Xiaolong Yang, Yawei Hao, Alex Ferenczy, Shou-Ching Tang, and Alan Pater, pages 200–207 (doi:10.1006/excr.1998.4349): On page 206, the acknowledgments are incorrect. The correct acknowledgments should read:

We thank G. Chernenko for excellent technical assistance. The investigation was supported by grants from the Medical Research Council of Canada and a grant from the National Cancer Institute of Canada with funds from the Canadian Cancer Society.